

## Cancer Associated Plexin B1 Mutations

This application is the U.S. national phase of international application PCT/GB2003/005223, filed 28 November 2003, which  
5 designated the U.S. and claims benefit of GB 0227908.1, filed 29 November 2002, the entire contents of each of which are incorporated herein by reference.

This invention relates to the identification of cancer-  
10 associated mutations in components of the semaphorin signalling pathway, in particular in the plexinB1 transmembrane receptor.

Semaphorins were first identified as extra-cellular cues for axon guidance in the nervous system and have since been shown  
15 to have roles in cell-cell communication and cell migration in a variety of adult and embryonic tissues (Kolodkin, A. L. et al. Cell 75 7 (1993): 1389-99; Kolodkin, A. L. Prog. Brain Res. 117 (1998): 115-32; Zou, Y. et al. Cell 102 3 (2000): 363-75).

20 Semaphorin signalling is mediated by transmembrane receptors called plexins, which can be grouped into 4 sub-families, plexin-A, B, C and D (Tamagnone, L. and P. M. Comoglio. Trends Cell Biol. 10 9 (2000): 377-83; Tamagnone, L. et al. Cell 99 1 (1999): 71-80: WO01/14420).

25 PlexinB1 has been shown to interact with a variety of factors, including semaphorin 4D, c-Met, neuropilins, active Rac1 and the guanine nucleotide exchange factors (GEFS), PDZ-RhoGEF and LARG (Kolodkin, A. L. et al. Cell 90.4 (1997):

753-62; Hirotsani, M. et al. Biochem. Biophys. Res. Commun.  
297.1 (2002): 32-37; Vikis, H. G. et al. Proc. Natl. Acad.  
Sci. U.S.A 97 23 (2000) 12457-62; Driessens, M. H. et al.  
Curr.Biol. 11.5 (2001) 339-44; Driessens, M. et al. FEBS  
5 Lett. 529.2-3 (2002) 168; Aurandt, J. et al. Proc. Natl.  
Acad. Sci. U.S.A 99 19 (2002) 12085-90; Perrot, V. et al  
J.Biol.Chem. 277.45 (2002) 43115-20; Swiercz, J. M. et al.  
Neuron 35.1 (2002): 51-63). Despite these interactions, the  
exact function of plexinB1 is not yet known.

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The present inventors have now identified mutations in  
plexinB1 that are closely associated with cancer. The  
frequency of these mutations, in particular in prostate and  
breast cancer, indicates that they may be useful in screening  
15 and diagnosis of cancer and as drug targets in the  
development of anti-cancer therapeutics.

One aspect of the invention provides a method of assessing an  
individual for a cancer condition comprising;

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providing a tissue sample obtained from said individual,  
and;

determining the presence in said sample of one or more  
cells comprising a plexinB1 nucleic acid sequence having one  
or more mutations therein.

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The presence of one or more cells comprising a mutant  
plexinB1 nucleic acid sequence in a sample obtained from an  
individual may be indicative of said individual having or  
being at risk of having a cancer condition. The one or more  
30 mutations in the nucleic acid sequence may alter the

expression and/or activity of the encoded plexinB1 polypeptide.

A cancer condition as described herein may include any type of solid cancer and malignant lymphoma and especially leukaemia, sarcomas, skin cancer, bladder cancer, breast cancer, uterus cancer, ovary cancer, prostate cancer, lung cancer, colorectal cancer, cervical cancer, liver cancer, head and neck cancer, oesophageal cancer, pancreas cancer, renal cancer, stomach cancer and cerebral cancer. In particular, methods of the invention may be useful in the treatment of breast or prostate cancer.

An individual may be healthy or may be suspected of or at risk of suffering from a cancer condition. In other embodiments, an individual may be suffering from a cancer condition. In some embodiments, an individual may be undergoing a cancer treatment and methods of the invention may be useful in determining the progress of the treatment.

A sample obtained from an individual may be a tissue sample comprising one or more cells, for example a biopsy from a cancerous tissue as described above, or, in certain contexts, a non-cancerous tissue.

Methods of the invention may be useful in characterising a cancer condition, for example for prognostic purposes. For example, the presence of a plexinB1 nucleic acid or polypeptide having one or more mutations as described herein may indicate that the cancer condition is an invasive or

metastatic cancer condition e.g. a cancer condition which is prone to or has a high probability of metastasis. In some embodiments, a method may comprise determining the level or amount of cells having a plexinB1 mutation in sample, for example a sample from a primary tumour. An increased level of cells in the sample comprising a mutant plexinB1 sequence may be indicative that the cancer condition is invasive or susceptible to metastasis.

A mutant plexinB1 nucleic acid may comprise a nucleotide sequence which has one or more mutations relative to the wild-type plexinB1 nucleotide sequence, as set out in AB007867. The mutations may be deletions, insertions or substitutions of one or more nucleotides. The one or more mutations may be in a coding or non-coding region of the plexin nucleic acid sequence and may alter the expression or function of the plexinB1 polypeptide. In other words, the mutant nucleic acid may encode a mutant plexinB1 polypeptide sequence with aberrant activity, or may encode a wild-type plexinB1 polypeptide which is expressed at an aberrant e.g. an increased or reduced, level, for example by means of an alteration in the activity of a plexinB1 regulatory element. A mutant nucleic acid may have one, two, three, four or more mutations relative to the wild-type sequence.

In some embodiments, the one or more mutations may occur in the region of the nucleic acid which encodes the cytoplasmic domain of the plexinB1 polypeptide, for example in the nucleotide sequence corresponding to exons 22, 23, 24, 25, 26, 27, 28 or 29. In some embodiments, a mutation may be in

the Rac1 binding region (i.e. amino acid residues 1724-1870: Driessens et al (2001) Curr Biol 11 339-344). Such a nucleic acid sequence may have a mutation at one or more mutation positions selected from the group consisting of 5059, 5060, 5 5074, 5107, 5359, 5401, 5452, 5458, 5468, 5474, 5596, 5653, 5662, 5674, 5713, 5714 and 5980 relative to the wild-type plexinB1 coding sequence. The mutation may be selected from the group consisting of C5059T, C5060T, G5074A, A5107G, A5359G, T5401A, G5452A, G5458A, T5468C, A5474G, A5596G, 10 A5653G, C5662T, A5674G, C5713T, T5714C and C5980T.

The nucleic acid sequence may be a genomic sequence, for example a genomic sequence comprising one or more of exons 22, 23, 24, 25, 26, 27, 28 and 29, or may be an RNA or cDNA 15 sequence.

As described above, a mutant plexin nucleic acid sequence may encode a plexinB1 polypeptide having one or more mutations therein. A method of assessing an individual for a cancer 20 condition according to the invention may thus comprise; providing a tissue sample obtained from the individual, and; determining the presence in the sample of one or more cells comprising a mutant plexinB1 polypeptide. The presence of one or more such cells may be indicative of said individual 25 having or being at risk of having a cancer condition.

A mutant plexinB1 polypeptide may comprise an amino acid sequence which has one or more mutations relative to the wild-type plexinB1 sequence set out in AB007867. The 30 mutations may be deletions, insertions or substitutions of

one or more amino acids. In some embodiments, the mutations may occur in the cytoplasmic domain of the plexinB1 polypeptide. A mutant polypeptide may have one, two, three, four or more mutations relative to the wild-type sequence.

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The one or more mutations may, for example, occur at a mutation site in the plexinB1 amino acid sequence selected from the group consisting of K1613, T1697, G1728, A1730, T1733, T1776, T1795, T1802, P1597, P1798, F1711, G1602,

10 L1815, N1735 and R1904. Suitable mutations include substitutions selected from the group consisting of T1697A, G1728S, A1730, T1733I, T1776A, T1795A, T1802A, P1597L, P1597S, P1798S, F1711I, G1602T, L1815P, L1815F, K1613E, N1735S and R1904W.

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Amino acid residues in the plexinB1 sequence are numbered herein in a N to C direction, starting at the initiating Met residue, which is numbered 1, as set out in AB007867.1

20 The presence of the one or more cells in the test sample may be determined by detecting the presence of a nucleic acid sequence encoding the mutant plexinB1 polypeptide or by detecting the presence of the mutant plexinB1 polypeptide.

25 Various methods are available for determining the presence or absence in a test sample of a particular nucleic acid sequence, for example a nucleic acid sequence which has a particular nucleotide at a site of mutation. Furthermore, having sequenced nucleic acid of an individual or sample, the  
30 sequence information can be retained and subsequently

searched without recourse to the original nucleic acid itself. Thus, for example scanning a database of sequence information using computer or other electronic technology may identify a sequence alteration or mutation.

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Methods according to some aspects of the present invention may comprise determining the binding of an oligonucleotide probe to nucleic acid obtained from the sample, for example, genomic DNA, RNA or cDNA. The probe may comprise a nucleotide  
10 sequence which binds specifically to a nucleic acid in the presence of one or more mutations and does not bind specifically to the nucleic acid in the absence of the one or more mutations or vice versa.

15 The oligonucleotide probe may comprise a label and binding of the probe may be determined by detecting the presence of the label.

A method may include hybridisation of one or more (e.g. two)  
20 oligonucleotide probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving  
25 PCR. An example procedure would be a combination of PCR and low stringency hybridisation.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal  
30 of those skilled in the art. For instance, probes may be

radioactively, fluorescently or enzymatically labelled.  
Other methods not employing labelling of probe include  
examination of restriction fragment length polymorphisms,  
amplification using PCR, RN'ase cleavage and allele specific  
5 oligonucleotide probing. Probing may employ the standard  
Southern blotting technique. For instance, DNA may be  
extracted from cells and digested with different restriction  
enzymes. Restriction fragments may then be separated by  
electrophoresis on an agarose gel, before denaturation and  
10 transfer to a nitrocellulose filter. Labelled probe may be  
hybridised to the DNA fragments on the filter and binding  
determined.

Nucleic acid may be screened using a variant- or allele-  
15 specific probe. Such a probe may correspond in sequence to a  
region of the plexinB1 gene, or its complement, which  
contains one or more of the mutations described herein, which  
are shown to be associated with cancer. Under suitably  
stringent conditions, specific hybridisation of such a probe  
20 to nucleic acid from a sample is indicative of the presence  
of the mutation in the test nucleic acid. For efficient  
screening purposes, more than one probe may be used on the  
same test sample.

25 Those skilled in the art are well able to employ suitable  
conditions of the desired stringency for selective  
hybridisation, taking into account factors such as  
oligonucleotide length and base composition, temperature and  
so on.



Suitable selective hybridisation conditions for oligonucleotides of 17 to 30 bases include hybridisation overnight at 42°C in 6X SSC and washing in 6X SSC at a series of increasing temperatures from 42°C to 65°C.

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Other suitable conditions and protocols are described in Molecular Cloning: a Laboratory Manual: 3rd edition, Sambrook & Russell, 2001, Cold Spring Harbor Laboratory Press NY and Current Protocols in Molecular Biology, Ausubel et al. eds. John Wiley & Sons, 1992.

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Nucleic acid, which may be genomic DNA, RNA or cDNA, or an amplified region thereof, may be sequenced to identify or determine the presence of a mutation therein. A mutation may be identified by comparing the sequence obtained with the nucleotide sequence of AB007867. A mutation may be a deletion, substitution or insertion of one or more nucleotides relative to the AB007867 sequence. In particular, the presence of one or more mutations identified herein to be associated with cancer may be determined.

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Sequencing may be performed using any one of a range of standard techniques. Sequencing of an amplified product may, for example, involve precipitation with isopropanol, resuspension and sequencing using a TaqFS+ Dye terminator sequencing kit. Extension products may be electrophoresed on an ABI 377 DNA sequencer and data analysed using Sequence Navigator software.

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Since it will not generally be time- or labour-efficient to sequence all nucleic acid in a sample or even the whole PlexinB1 gene or coding sequence, a specific amplification reaction such as PCR using one or more pairs of primers may conveniently be employed to amplify the region of interest within the nucleic acid sequence, for example, the portion of the sequence suspected of containing cancer associated mutations. The amplified nucleic acid may then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular feature.

Suitable amplification reactions include the polymerase chain reaction (PCR) (reviewed for instance in "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, 1990, Academic Press, New York, Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, and Ehrlich et al, Science, 252:1643-1650, (1991)). PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid used as template in the amplification reaction may be genomic DNA.

Other specific nucleic acid amplification techniques include strand displacement activation, the Q $\beta$  replicase system, the repair chain reaction, the ligase chain reaction, rolling circle amplification and ligation activated transcription. For convenience, and because it is generally preferred, the term PCR is used herein in contexts where other nucleic acid amplification techniques may be applied by those skilled in

the art. Unless the context requires otherwise, reference to PCR should be taken to cover use of any suitable nucleic amplification reaction available in the art.

- 5 Methods of the present invention may therefore comprise amplifying a portion of the plexinB1 coding sequence containing one or more sites of mutation from one or more cells from said tissue sample.
- 10 Mutation- or variant-specific oligonucleotides may be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may  
15 for instance be treated in a way that enables one to display the polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected.
- 20 An oligonucleotide for use in nucleic acid amplification may be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length, but need not be more than 18-20.
- 25 In some embodiments, the region of nucleic acid sample comprising a position of mutation may be amplified using a pair of oligonucleotide primers, of which the first member of the pair comprises a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' of the  
30 position of mutation, and the second member of the primer

pair comprises a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 3' of the position of mutation.

- 5 Mutation sites within the plexin B1 nucleotide sequence are discussed elsewhere herein.

In other embodiments, the first member of the pair of oligonucleotide primers may comprise a nucleotide sequence  
10 which hybridises to a complementary sequence which is proximal to and 5' or 3' of the mutation site, and the second member of the pair may comprise a nucleotide sequence which hybridises under stringent conditions to a sequence which includes a particular nucleotide (i.e. A, G, T or C) at the  
15 mutation site and not to sequences which include other nucleotides at the mutation site, such that amplification only occurs in the presence of the particular nucleotide at the mutation site.

- 20 Another aspect of the invention provides a pair of oligonucleotide amplification primers suitable for use in the methods described herein.

A suitable pair of amplification primers according to this  
25 aspect may have a first member comprising a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' of a mutation site in the plexinB1 coding sequence, for example a mutation site in the sequence encoding the cytoplasmic domain of the polypeptide, such as

at position 5059, 5060, 5074, 5107, 5359, 5401, 5452, 5458,  
5468, 5474, 5596, 5653, 5662, 5674, 5713, 5714 or 5980, and;  
a second member comprising a nucleotide sequence which  
hybridises to a complementary sequence which is proximal to  
5 and 3' of the mutation site.

The first member may hybridise to a first nucleic acid strand  
having a sequence as shown in AB007867, and the second member  
may hybridise to a strand complementary to the first strand.

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The identity of the nucleotide at the position of mutation  
may then be determined by determining the binding of an  
oligonucleotide probe to the amplified region. A suitable  
oligonucleotide probe comprises a nucleotide sequence that  
15 binds specifically to a sequence comprising a particular  
nucleotide at the mutation site and does not bind  
specifically to other sequences comprising other residues at  
the mutation site.

20 Other suitable pairs of amplification primers may have a  
first member comprising a nucleotide sequence which  
hybridises to a complementary sequence which is proximal to  
and 5' or 3' of a mutation site, for example a mutation site  
in the sequence encoding the cytoplasmic domain of the  
25 polypeptide, such as at position 5059, 5060, 5074, 5107,  
5359, 5401, 5458, 5452, 5468, 5474, 5596, 5653, 5662, 5674,  
5713, 5714 or 5980 of the plexinB1 coding sequence, and a  
second member of the pair comprising a nucleotide sequence  
which hybridises under stringent conditions to a sequence  
30 comprising a particular nucleotide at the mutation site and

not to sequences having other nucleotides at the mutation site, such that amplification only occurs in the presence of the particular nucleotide.

5 An alternative or supplement to looking for the presence of mutant sequences in a test sample is to look for the presence of the normal, wild type sequence, e.g. using a suitably specific oligonucleotide probe or primer. Use of oligonucleotide probes and primers has been discussed in more  
10 detail above.

A further aspect of the present invention provides an oligonucleotide which hybridises specifically to a nucleic acid sequence which comprises a particular nucleotide at a  
15 mutation site within the plexinB1 nucleic acid sequence, for example within the coding sequence at a position selected from the group consisting of 5059, 5060, 5074, 5107, 5359, 5401, 5452, 5458, 5468, 5474, 5596, 5653, 5662, 5674, 5713, 5714 and 5980.

20 Such oligonucleotides may be used in a method of screening nucleic acid. Some preferred oligonucleotides have a sequence which is complementary to the plexinB1 coding sequence, or a sequence which differs from such a sequence by  
25 addition, substitution, insertion or deletion of one or more nucleotides, but preferably without abolition of ability to hybridise selectively to a sequence comprising a particular residue (i.e. one of A, G, C or T) at a mutation site as described herein, that is wherein the degree of similarity of

the oligonucleotide or polynucleotide with one of the sequences given is sufficiently high.

In some preferred embodiments, oligonucleotides according to  
5 the present invention are at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length. Oligonucleotides may be up to about 100 nucleotides in length, more preferably up to about 50 nucleotides in  
10 length, more preferably up to about 30 nucleotides in length.

Approaches that rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH  
15 etc.), an oligonucleotide probe will hybridise with a sequence that is not entirely complementary. The degree of base pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis-  
20 match between two annealing nucleic acid molecules.

For instance, RN'ase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresing test nucleic acid to which the relevant probe or probe has annealed and  
25 looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full-length probe/test hybrid.

Thus, an oligonucleotide probe that has the sequence of a  
30 region of the normal plexinB1 coding sequence (either sense

or anti-sense strand) in which the mutations associated with cancer or cancer susceptibility as described herein are known to occur, (e.g. the sequence encoding the cytoplasmic domain) may be annealed to test nucleic acid and the presence or  
5 absence of a mis-match determined.

Detection of the presence of a mis-match may indicate the presence in the test nucleic acid of a mutation associated with cancer or cancer susceptibility. On the other hand, an  
10 oligonucleotide probe that has the sequence of a region of the gene including a mutation associated with cancer or cancer susceptibility may be annealed to test nucleic acid and the presence or absence of a mis-match determined. The presence of a mis-match may indicate that the nucleic acid in  
15 the test sample has the normal sequence (the absence of a mis-match indicating that the test nucleic acid has the mutation). In either case, a battery of probes to different regions of the gene may be employed.

20 Oligonucleotide probes and primers based on the sequence of plexinB1 may be designed by the ordinary skilled person using conventional primer design software. Those skilled in the art are well versed in the design of suitable primers for use processes such as PCR. Various techniques for synthesizing  
25 oligonucleotide primers are well known in the art, including phosphotriester and phosphodiester synthesis methods.

Hybridisation with allele specific oligonucleotides may be conveniently carried out using an oligonucleotide array,



preferably a microarray, to determine the identity of a nucleotide present at one or more positions of mutation.

Microarrays are particularly suitable for the detection of polymorphisms (Yershov, G. et. al.(1996) PNAS USA, Genetics, Vol. 93, 4913-4918; Schena, M., 1999, DNA Microarrays "a practical approach", ISBN, 0-19-963777-6, Oxford press, editor B. D. Hames; Cheung, V. G., et. al., 1999, Nat. Genet., vol. 21, 15-19, WO84/01031, WO88/1058, WO89/01157, WO93/8472, WO95/18376/ WO95/18377, WO95/24649 and EP-A-0373203).

In brief, the DNA microarray may be generated using oligonucleotides that have been selected to hybridise with the specific target mutation or polymorphism. These oligonucleotides may be applied by a robot onto a predetermined location of a glass slide, e.g. at predetermined X, Y cartesian coordinates, and immobilised. An amplified genomic product (e.g. fluorescently labelled DNA) is introduced on to the DNA microarray and a hybridisation reaction conducted so that sample RNA or DNA binds to complementary oligonucleotide sequences in a sequence-specific manner, and unbound material is washed away. Amplified sample DNA containing a mutation can thus be detected by its binding to complementary oligonucleotides on the array to produce a signal. The absence of a signal for a specific oligonucleotide probe indicates that the amplified sample does not have the corresponding mutation. The signal produced at each coordinate on the microarray is conveniently

read using an automated detector in order to correlate each signal with a particular oligonucleotide.

Nucleic acid, such as an oligonucleotide probe and/or pair of  
5 amplification primers, may be provided as part of a kit for performing a method according to the invention, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or  
10 a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be  
15 labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing or preparing the test sample itself.

20 Another aspect of the present invention provides a method for determining the presence or absence of a specific nucleotide at a mutation site in the plexinB1 nucleic acid sequence in a test sample comprising:

contacting a plexinB1 nucleic acid sequence with a probe  
25 which specifically binds to a mutant plexinB1 nucleotide sequence, for example a nucleotide sequence having a particular residue at a mutation site (i.e. a mutant plexinB1 sequence), such as a sequence encoding a mutant plexinB1 polypeptide as described herein; and,

determining binding of the nucleic acid sequence and the probe.

The nucleic acid sequence may comprise one or more mutation  
5 sites selected from the group consisting of 5059, 5060, 5074,  
5107, 5359, 5401, 5452, 5458, 5468, 5474, 5596, 5653, 5662,  
5674, 5713, 5714 and 5980 of the plexinB1 coding sequence.  
The identity of the nucleotide at the one or more sites of  
mutation determines the sequence of the mutant plexinB1  
10 nucleic acid and the binding of the probe.

A method for determining the presence or absence in a test  
sample of a mutation within a test plexinB1 nucleic acid  
sequence may comprise:

15 determining the identity of the nucleotide at one or  
more positions of mutation in the test sequence,  
the presence of a mutation in the test plexinB1 sequence  
being inferred by the identity of the nucleotide at the one  
or more positions of mutation.

20 The nucleotide at the one or more positions of mutation in  
the test sequence may be compared with the nucleotide at the  
corresponding one or more positions of mutation in the wild  
type plexinB1 sequence. The presence of a different  
25 nucleotide in the test sequence compared to the wild type  
sequence at the one or more positions is indicative of the  
presence of a mutation.

A suitable position of mutation may include a position  
30 selected from the group consisting of positions 5059, 5060,

5074, 5107, 5359, 5401, 5452, 5458, 5468, 5474, 5596, 5653, 5662, 5674, 5713, 5714 and 5980 of the plexinB1 coding sequence.

- 5 A mutation may be a cancer-associated mutation as described herein.

The test sample may be a sample of genomic DNA, cDNA or RNA from a tissue sample obtained from an individual, for example  
10 a tumour or other tissue biopsy or a sample of biological fluid, such as a blood sample. The individual may be healthy or may be suffering from a cancer or other condition as described herein.

- 15 Optionally, such a method may comprise amplifying the plexinB1 nucleic acid sequence using a pair of oligonucleotide primers. As noted, physical detection may be employed using for example hybridisation of a suitable probe, or a transcription factor or other agent that binds nucleic  
20 acid in a sequence-specific fashion, or detection may be performed *in silico* using suitable data analysis techniques, e.g. on a computer.

The identity of the nucleotide at a site of mutation may be  
25 determined using a method described herein, in particular, the presence of the nucleotide other than the nucleotide in the corresponding wild-type sequence may be determined.

Mutations associated with cancer may also be detected at the  
30 protein level by detecting the presence of a mutant plexinB1

polypeptide. For practical purposes, or at least commercial purposes bearing in mind cost and time, assessment of target protein expression at the protein level may be preferred over assessment at the nucleic acid level.

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A method of determining the presence, absence or level of cancer cells in a sample from an individual, may include contacting a sample with a specific binding member directed against a mutant plexiB1 polypeptide, and determining binding  
10 of the specific binding member to the sample. Binding of the specific binding member to the sample may be indicative of the presence of a cancer cell within the sample.

Preferred specific binding molecules for use in aspects of  
15 the present invention include antibodies and fragments or derivatives thereof ('antibody molecules'). Antibody molecules are described in more detail below.

A cancer cell may be a cell from any type of cancer, for  
20 example as described above. In some preferred embodiments, the cancer cell may be a prostate cancer cell or a breast cancer cell.

A mutant plexinB1 polypeptide may comprise one or more  
25 mutations, for example one, two, three or more mutations, relative to the wild-type sequence. In some embodiments, the one or more mutations may occur within the cytoplasmic domain of the polypeptide. Suitable mutations may occur at mutation sites T1697, G1728, A1730, K1613, T1733, T1776, T1795, T1802,  
30 P1597, P1798, F1711, G1602, L1815, N1735 and R1904 in the

plexinB1 amino acid sequence and may, for example, include one or more substitutions selected from the group consisting of T1697A, G1728S, A1730T, K1613E, T1733I, T1776A, T1795A, T1802A, P1597L, P1597S, P1798S, F1711I, G1602T, L1815F, L1815P, N1735S and R1904W.

In particular, mutations in plexinB1 nucleic acid and polypeptide sequences are associated with invasive cancers which are prone to metastasise and methods of the invention may be used to determine the presence of invasive cancer cells in a sample obtained from an individual.

Another aspect of the present invention provides for a method of categorising a tissue as (i) normal, (ii) potentially or actually pre-cancerous or cancerous, dysplastic, or neoplastic or (iii) cancerous and prone to metastasise, the method including determining binding to a sample of the tissue of a specific binding member directed against a mutant plexinB1 polypeptide. The pattern or degree of binding may be compared with that for a known normal sample and/or a known abnormal sample.

A method of the invention may be used to characterise a cell, for example a cancer cell, as invasive or non-invasive, for example in the prognosis of a cancer condition. Thus, binding of (e.g.) an anti-mutant plexinB1 specific binding member to a sample provides for categorising the tissue from which the sample is derived as potentially or actually pre-cancerous or cancerous, dysplastic or neoplastic or cancerous and potentially metastatic. The method may be used to pre-screen samples before further analysis. The method may also

be useful for screening or analysis of samples previously tested using another technique.

A specific binding molecule, for example an antibody molecule, may be provided in a kit, which may include instructions for use in accordance with a method of the invention. Such kits are provided as a further aspect of the invention. One or more other reagents may be included, such as labelling molecules, and so on (see below). Reagents may be provided within containers, which protect them from the external environment, such as a sealed vial. A kit may include one or more articles for providing or preparing the test sample itself, depending on the tissue of interest. A kit may include any combination of, or all of, a blocking agent to decrease non-specific staining, a storage buffer for preserving binding molecule activity during storage, staining buffer and/or washing buffer to be used during antibody staining, a positive control, a negative control and so on. Positive and negative controls may be used to validate the activity and correct usage of reagents employed in accordance with the invention and which may be provided in a kit. Controls may include samples, such as tissue sections, cells fixed on coverslips and so on, known to be either positive or negative for the presence of the mutant plexinB1. The design and use of controls is standard and well within the routine capabilities of those of ordinary skill in the art.

Samples to be subjected to a contact with a binding member in accordance with methods of the invention may be prepared using any available technique which allows binding of a

specific binding molecule to the mutant plexinB1 polypeptide. Various techniques are standard in the art.

The reactivities of a binding member such as an antibody on normal and test samples may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding binding molecule (e.g. antibody) and reporter molecule.

One favoured mode is by covalent linkage of each binding member with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine. Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes that catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or



emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed. Further examples are horseradish peroxidase and chemiluminescence.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Methods of assessing a cancer condition as described herein may be employed before, during and/or after a course of cancer treatment in order to determine the progress and/or effectiveness of the treatment.

A further aspect of the invention provides an antibody molecule that binds specifically to mutant plexinB1 polypeptide. Such an antibody binds preferentially to mutant plexinB1 polypeptide relative to wild-type plexinB1 polypeptide.

Antibody molecules may be useful both in the diagnosis and therapy of cancer, in accordance with the invention.

Antibodies that are specific for a mutant plexinB1 polypeptide may be obtained using techniques that are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep, monkey or bird such as chicken) with the mutant

protein or a fragment thereof, or a cell or virus that expresses the protein or fragment.

Antibodies may be obtained from immunised animals using any  
5 of a variety of techniques known in the art, and screened, preferably using binding of antibody to mutant plexinB1, or fragments comprising the cytoplasmic domain thereof. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

10

The production of specific monoclonal antibodies is also well established in the art.

As an alternative or supplement to immunising a mammal with a  
15 peptide, an antibody specific for a target may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces;  
20 for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with the target or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest (or a fragment  
25 thereof).

An antibody molecule which is specific for a mutant plexinB1 may be conjugated or bound to a cytotoxic agent. Binding of the antibody molecule to the mutant plexinB1 may be used to

selectively target the cytotoxic agent to a cancer cell. Many suitable cytotoxic agents are known in the art.

Another aspect of the invention provides a method of

- 5 identifying and/or obtaining an antibody specific for a mutant plexinB1, the method comprising;
- providing a population of antibody molecules specific for mutant plexinB1,
  - contacting said population with a normal plexinB1
  - 10 polypeptide,
  - identifying one or more members of said population which bind preferentially to mutant plexinB1 relative to normal plexinB1

- 15 A method may include isolating and/or purifying said one or more members. A population of antibody molecules specific for mutant plexinB1 may be obtained as described above.

- Antibodies may be modified in a number of ways. Indeed,
- 20 unless context precludes otherwise, the term "antibody" should be construed as covering any specific binding substance having an antibody antigen-binding domain. Thus, this covers antibody fragments, derivatives, and functional equivalents, including any polypeptide comprising an
- 25 immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

30

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')<sub>2</sub> fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Recombinant expression of polypeptides, including antibody molecules, is well-known in the art.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and many others. A common, preferred bacterial host is E. coli. The preferred hosts for baculovirus expression are insect cells such as the SF9 cell line.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 3rd edition, Sambrook & Russell 2001, Cold Spring Harbor Laboratory Press. Transformation

procedures suitable for different hosts are well known in the art.

Following production by expression from encoding nucleic acid an antibody or other specific binding molecule directed against mutant or wild-type plexinB1, may be recovered and may be isolated, if necessary conjugated to an appropriate label or reporter, and provided for use in assessing a cancer condition in an individual as described herein.

Determination of binding to mutant plexinB1 polypeptide *in vivo* may be used to identify localisations of cancer cells in the body. Labelled binding molecules against mutant plexinB1 may be administered to an individual and binding within the body determined. When a radionucleotide such as Iodine-125, Indium-111, Thallium-201 or Technetium-99m is attached to an antibody, and the antibody localises preferentially in tumour rather than normal tissues, the presence of radiolabel in tumour tissue can be detected and quantitated using a gamma camera or scintigraphy. Radiolabelling with technetium-99m is described in Pak et al (1992), Nucl. Med. Biol. 19; 699-677. A review of cancer imaging with anti-CEA antibodies is provided by Goldenberg D.M., Int. J. of Biol. Markers 1992, 7; 183-188. The present invention of course extends to specific binding members directed against mutant plexinB1 as disclosed, for use in any such *in vivo* method.

As described above, mutations in the plexinB1 nucleic acid and amino acid sequences have further been shown by the inventors to be associated with the invasiveness of a cancer

cell. Thus, methods of the invention may be useful in the prognosis of an individual having a cancer condition.

Another aspect of the invention provides a method of  
5 determining the invasiveness of a cancer cell from a sample obtained from an individual, the method comprising,

determining the presence or absence in said cell of a plexinB1 nucleic acid having one or more mutations therein, the presence of said plexinB1 nucleic acid being indicative  
10 that the cancer cell is invasive.

Mutant plexinB1 nucleic acid is described in detail above. In particular, a mutant plexinB1 nucleic acid from an invasive cancer cell may have two or more, or three or more mutations  
15 relative to the wild-type sequence. The cancer cell may be a prostate cancer cell or other cancer cell as described above.

In some embodiments a mutant plexinB1 nucleic acid from an invasive cancer cell may include one or more of the mutations  
20 T1795A, P1597L, P1597S and L1815P. In some preferred embodiments, a mutant plexinB1 includes the mutation T1795A.

The presence of a mutant plexinB1 nucleic acid may be determined by detecting the presence of a polypeptide  
25 sequence encoded by said mutant plexinB1 polypeptide or by directly detecting the mutant plexinB1 nucleic acid, as described above.

The results set out herein show that the proportion of cells containing plexinB1 mutations is increased in metastatic tumours compared to primary tumours.

5 A method of determining the invasiveness or susceptibility to metastasis of a cancer condition in an individual may comprise;

determining the presence, level or amount of cancer cells which comprise a plexinB1 nucleic acid sequence having one or more mutations therein in a sample obtained from said individual.

The presence of said cells in the sample or the presence of an elevated level of said cells relative to cells with wild-type plexinB1 may be indicative that the cancer condition is susceptible to or at risk of metastasis.

Another aspect of the invention provides a method of identifying and/or obtaining a compound as a putative anti-cancer agent, the method comprising;

contacting a plexinB1 polypeptide with a test compound, and;

determining the activity of the plexinB1 polypeptide in the presence relative to the absence of test compound.

A decrease in activity in the presence relative to the absence of test compound is indicative that the compound is a putative anti-cancer agent.

The activity of the plexinB1 polypeptide may be determined by determining the binding of said polypeptide to a ligand such as semaphorin 4D, neuropilin, c-Met, active Rac1, PDZ-RhoGEF or LARG and/or by determining the activation of Rho A or the  
5 binding of Rho A to effector proteins (e.g. ROCK).

In some embodiments, the plexinB1 polypeptide may be a mutant plexinB1 polypeptide as described above. In an optional step following the identification of a test compound which alters  
10 the activity of the mutant plexinB1 polypeptide, the effect of the test compound on wild-type plexinB1 may be determined. A method according to such embodiments may comprise contacting a wild-type plexinB1 polypeptide with the test compound, and; determining the activity of the wild-type  
15 plexinB1 polypeptide.

In some embodiments, a putative anti-cancer agent may disrupt the interaction of a mutant plexinB1 polypeptide with a wild-type polypeptide. A method of the invention may comprise the  
20 steps of;

contacting the mutant plexinB1 polypeptide with the test compound in the presence of a wild-type plexinB1, and;

determining the activity of the wild-type plexinB1 polypeptide.

25

A test compound which preferentially inhibits the activity of mutant plexinB1 relative to wild type plexinB1 may be particularly useful in specifically targeting cancer cells.



In other embodiments, the plexinB1 polypeptide may be a wild-type plexinB1 polypeptide. A test compound may be identified which alters the activity of both the wild-type and mutant plexinB1 polypeptide. Such a compound may be particularly  
5 useful in treating cancer when specifically targeted to a tumour site, for example using tumour-specific antibodies.

An agent identified using one or more primary screens (e.g. in a cell-free system) as having ability to modulate the  
10 activity of plexinB1 may be assessed further using one or more secondary screens. A secondary screen may involve testing for effects on growth of cancer cells in vitro, in particular anchorage independent growth, or a biological function of plexinB1 or semaphorin signalling, for example in  
15 an animal model.

Suitable biological functions which may be assessed in a secondary screen include reduction in size or number of tumours, inhibition of metastasis, or a reduction in other  
20 symptoms or effects of a cancer condition.

In some embodiments, the activity of the plexinB1 polypeptide may be determined by measuring the tumourigenicity in an animal model of cancer cells, such as NIH3T3 cells, which  
25 express the plexinB1 polypeptide. Suitable animal models include athymic nude mice. A reduction in the rate of tumour production in the presence relative to the absence of test compound may be indicative that the compound is a putative anti-cancer agent which enhances or restores the tumour  
30 suppressor activity of plexinB1.

In some embodiments, test compounds may be screened initially using a secondary screen as described above, without an initial primary screening step.

5

One class of putative inhibitor compounds can be derived from the plexinB1 polypeptide, in particular the mutant plexinB1 polypeptide, and/or ligands which bind to it, including semaphorin 4D, active Rac1, neuropilin, PDZ-RhoGEF and LARG.

10 Membrane permeable peptide fragments of from 5 to 40 amino acids, for example, from 6 to 10 amino acids may be tested for their ability to disrupt such interaction or activity. In some embodiments, peptide fragments may comprise the cytoplasmic domain of plexinB1, in particular one or more  
15 mutation sites within the cytoplasmic domain, as described herein.

Peptide fragments may also be generated wholly or partly by chemical synthesis. The compounds of the present invention  
20 can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical  
25 Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any  
30 combination of solid-phase, liquid phase and solution

chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

The inhibitory properties of a peptide fragment as described above may be increased by the addition of one of the following groups to the C terminal: chloromethyl ketone, aldehyde and boronic acid. These groups are transition state analogues for serine, cysteine and threonine proteases. The N terminus of a peptide fragment may be blocked with carbobenzyl to inhibit aminopeptidases and improve stability (Proteolytic Enzymes 2nd Ed, Edited by R. Beynon and J. Bond, Oxford University Press, 2001).

Another convenient way of producing polypeptide molecules, which may be full-length sequences or peptide fragments thereof, for use in methods of the invention, is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Antibodies which specifically bind to wild-type or mutant plexinB1 polypeptide, or regions thereof, form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which alter (i.e. enhance or disrupt) the interactions and/or activity of plexinB1.

Techniques for obtaining antibodies are standard in the art and are described elsewhere herein.

Antibody molecules may for example be micro-injected into cells, e.g. at a tumour site, subject to radio- and/or chemotherapy (as discussed already above). Antibodies may be employed in accordance with the present invention for other therapeutic and non-therapeutic purposes, which are discussed elsewhere herein.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential modulator (for example, inhibitor) compounds with particular molecular shape, size and charge characteristics.

A potential modulator compound may be a "functional analogue" of a peptide or other compound which modulates mutant plexinB1 activity. A functional analogue has the same functional activity as the peptide or other compound in question, i.e. it may alter (i.e. enhance or interfere with) the binding between mutant plexinB1 and one or more ligands. Examples of such analogues include chemical compounds which are modelled to resemble the three dimensional structure of the mutant or wild-type plexinB1 polypeptide, and in particular the arrangement of the key amino acid residues as they appear in the mutant form of plexinB1.

Mutant or wild-type plexinB1 polypeptide and binding partners may be used in methods of designing mimetics of these molecules suitable for inhibiting mutant plexinB1 activity.

- 5 Accordingly, the present invention provides a method of designing mimetics of wild-type or mutant plexinB1 polypeptide having the biological activity of modulating, e.g. enhancing or inhibiting, the activity of wild-type or mutant plexinB1 polypeptide, said method comprising:
- 10 (i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,
- (ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

15 Suitable modelling techniques are known in the art. This includes the design of so-called "mimetics" which involves the study of the functional interactions of the molecules and the design of compounds which contain functional groups

20 arranged in such a manner that they could reproduced those interactions.

The modelling and modification of a 'lead' compound to optimise its properties, including the production of

25 mimetics, is further described below.

As described above, the activity or function of a mutant plexinB1 may be inhibited, as noted, by means of a compound that interferes in some way with the interaction of plexinB1

30 with other factors described herein. An alternative approach

to inhibition employs regulation at the nucleic acid level to inhibit activity or function by down-regulating production of the mutant form of plexinB1.

A method of identifying and/or obtaining a compound which is a putative anti-cancer agent may comprise;

contacting a plexinB1 nucleic acid therein with a test compound, and;

determining the expression of the plexinB1 nucleic acid in the presence relative to the absence of test compound.

PlexinB1 nucleic acid sequences may be mutant plexinB1 sequences i.e. sequences having one or more mutations, or wild-type sequences. PlexinB1 nucleic acid sequences are described elsewhere herein. The plexinB1 nucleic acid may be within a cell, for example in a cancer cell line, such as a breast or prostate cancer cell line.

Expression of the nucleic acid may be determined using conventional techniques, such as Northern blotting or RT-PCR.

Anti-sense or RNAi technology may be used to inhibit the expression of a plexinB1 nucleic acid, in particular a mutant plexinB1 nucleic acid. The use of these approaches to down-regulate gene expression is now well-established in the art.

Methods of the present invention may include identifying the test compound as a putative anti-cancer agent. Methods may further include isolating, purifying, synthesising and/or

manufacturing a compound identified as a putative anti-cancer agent.

Optionally, compounds identified as putative anti-cancer agents using a method described herein may be modified to optimise activity or provide other beneficial characteristics such as increased half-life or reduced side effects upon administration to an individual.

Methods of the present invention may further include formulating a compound identified as a putative anti-cancer agent into a composition, such as a medicament, pharmaceutical composition or drug, with a pharmaceutically acceptable excipient as described below.

Another aspect of the invention provides a pharmaceutical composition comprising the compound as described above and a pharmaceutically acceptable excipient. Such a composition may be administered to an individual.

A method of making a pharmaceutical composition may comprise, identifying a compound as modulator of plexinB1, for example an inhibitor of mutant plexin B1 or an enhancer of wild-type plexin B1, using a method described herein, synthesising, preparing or isolating said modulator, admixing the modulator with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients to formulate or produce said composition; and, optionally, determining the activity of mutant plexinB1 as described herein in the presence of said composition.

In other embodiments, a method of producing a pharmaceutical composition may comprise;

5 identifying a compound which modulates the activity of a plexinB1 polypeptide using a method described herein; and, admixing the compound identified thereby with a pharmaceutically acceptable carrier.

The formulation of compositions with pharmaceutically acceptable carriers is described further below.

Another aspect of the invention provides a method for preparing a pharmaceutical composition, for example, for the treatment of a cancer condition comprising;

15 i) identifying a compound which is an agonist/antagonist of a plexinB1 polypeptide  
ii) synthesising the identified compound, and;  
iii) incorporating the compound into a pharmaceutical composition.

20

As described above, a plexinB1 polypeptide may be a wild-type or mutant plexinB1 polypeptide.

The identified compound may be synthesised using conventional chemical synthesis methodologies. Methods for the development and optimisation of synthetic routes are well known to a skilled person.

The compound may be modified and/or optimised as described above.

30



Incorporating the compound into a pharmaceutical composition may include admixing the synthesised compound with a pharmaceutically acceptable carrier or excipient.

5

The modification of a known pharmacologically active compound to improve its pharmaceutical properties is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. The design, synthesis and testing of modified active compounds, including mimetics, may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in modifying a compound which has a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g.

stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of plexinB1, in particular mutant plexinB1 as described herein, and ligands are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the modified compound is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade in vivo, while retaining the biological activity of the lead compound. Modified compounds found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final compounds for in vivo or clinical testing.

Those of skill in the art may vary the precise format of methods of the invention using routine skill and knowledge.

Aspects of the invention provide a compound obtained by a method as described above for use in a method of treatment and the use of such a compound in the manufacture of a medicament for use in the treatment of cancer.

5

Another aspect of the invention provides a method of treating a cancer condition, such as prostate cancer or breast cancer, in an individual, the method comprising inhibiting the activity of mutant plexinB1 polypeptide in one or more cells of said individual.

10

The activity or function of mutant plexinB1 polypeptide may be inhibited, for example, by administering an antagonist of mutant plexinB1 to said individual. A suitable antagonist may be an antibody molecule, peptide or small organic molecule, and may be obtained by a method described above.

15

As described above, an antagonist of mutant plexinB1 may be specific for mutant plexinB1 or may have an antagonistic effect on both wild-type and mutant plexinB1. An antagonist which acts on both wild-type and mutant plexinB1 may be specifically targeted to a tumour site using conventional techniques.

20

An alternative approach to inhibition employs regulation at the nucleic acid level to inhibit activity or function by down-regulating production of mutant plexinB1. The activity of mutant plexinB1 polypeptide may be inhibited by reducing or abolishing expression of plexin B1 polypeptide using anti-sense or RNAi technology.

30

Anti-sense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of mutant

5 plexinB1 polypeptide so that its expression is reduced or completely or substantially completely prevented. In addition to targeting coding sequence, anti-sense techniques may be used to target control sequences of a gene, e.g. in the 5' flanking sequence, whereby the antisense oligonucleotides can interfere with expression control  
10 sequences. The construction of antisense sequences and their use is described for example in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990) and Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992).

Oligonucleotides may be generated in vitro or ex vivo for  
15 administration or anti-sense RNA may be generated in vivo within cells in which down-regulation is desired. Thus, double-stranded DNA may be placed under the control of a promoter in a "reverse orientation" such that transcription of the anti-sense strand of the DNA yields RNA which is  
20 complementary to normal mRNA transcribed from the sense strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of  
25 action is still uncertain. However, it is established fact that the technique works.

The complete sequence corresponding to the coding sequence in reverse orientation need not be used. For example fragments of sufficient length may be used. It is a routine matter for

the person skilled in the art to screen fragments of various sizes and from various parts of the coding or flanking sequences of a gene to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A suitable fragment may have about 14-23 nucleotides, e.g. about 15, 16 or 17.

An alternative to anti-sense is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression; Angell & Baulcombe (1997) *The EMBO Journal* 16,12:3675-3684; and Voinnet & Baulcombe (1997) *Nature* 389: pg 553). Double stranded RNA (dsRNA) has been found to be even more effective in gene silencing than both sense or antisense strands alone (Fire A. et al *Nature*, Vol 391, (1998)). dsRNA mediated silencing is gene specific and is often termed RNA interference (RNAi).

RNA interference is a two-step process. First, dsRNA is cleaved within the cell to yield short interfering RNAs (siRNAs) of about 21-23nt length with 5' terminal phosphate and 3' short overhangs (~2nt). The siRNAs target the corresponding mRNA sequence specifically for destruction (Zamore P.D. *Nature Structural Biology*, 8, 9, 746-750, (2001))

RNAi may be also be efficiently induced using chemically synthesized siRNA duplexes of the same structure with 3'-overhang ends (Zamore PD et al. *Cell*, 101, 25-33, (2000)). Synthetic siRNA duplexes have been shown to specifically

suppress expression of endogenous and heterologous genes in a wide range of mammalian cell lines (Elbashir SM. et al. Nature, 411, 494-498, (2001))

Another possibility is that nucleic acid is used which on transcription produces a ribozyme, able to cut nucleic acid at a specific site - thus also useful in influencing gene expression. Background references for ribozymes include Kashani-Sabet and Scanlon, 1995, Cancer Gene Therapy, 2(3): 213-223, and Mercola and Cohen, 1995, Cancer Gene Therapy, 2(1), 47-59.

Thus, an inhibitor of mutant plexinB1 activity may comprise a nucleic acid molecule comprising all or part of the mutant or wild type plexinB1 coding sequence or the complement thereof

Such a molecule may suppress the expression of the mutant plexinB1 polypeptide and may comprise a sense or anti-sense mutant plexinB1 coding sequence or may be a mutant plexinB1 specific ribozyme, according to the type of suppression to be employed.

The type of suppression will also determine whether the molecule is double or single stranded and whether it is RNA or DNA.

A related aspect of the invention provides a method of treating a cancer condition, such as prostate cancer and breast cancer, in an individual, the method comprising increasing the activity of wild-type plexinB1 polypeptide in one or more cells of said individual.

Wildtype plexinB1 activity may be enhanced by increasing the level of plexinB1 polypeptide in the one or more cells, for example by expression from a recombinant plexinB1 coding sequence, or by administration of a plexinB1 agonist molecule.

Further aspects of the invention provide a nucleic acid encoding wild-type or mutant plexinB1 polypeptide, its complement or a fragment thereof for use in a method of treatment, for example in a method of treating cancer, for example prostate cancer and the use of nucleic acid encoding wild-type or mutant plexinB1, its complement or a fragment thereof in the manufacture of a medicament for the treatment of cancer. Such molecules may, for example, be useful in suppressing the expression of mutant plexinB1 or increasing the expression of wild-type plexin B1.

Mutations in the plexinB1 coding sequence are shown herein to be associated with anchorage-independent growth and metastasis. A method of reducing the invasiveness of a tumour in an individual may comprise inhibiting the activity of mutant plexinB1 polypeptide in one or more cells of said tumour, or enhancing the activity of wild-type plexinB1 polypeptide, as described above.

A compound may be administered in a precursor form, for conversion to an active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT; the former

involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see  
5 for example, EP-A-415731 and WO 90/07936).

Whether it is a polypeptide, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound that is to be given to an individual, administration is preferably  
10 in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of  
15 administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

20 A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present  
25 invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not  
30 interfere with the efficacy of the active ingredient. The



precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

5    Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils,  
10    mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

15    For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the  
20    art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

25

Aspects of the present invention will now be illustrated with reference to the following experimental exemplification, by way of example and not limitation. Further aspects and embodiments will be apparent to those of ordinary skill in

the art. All documents mentioned in this specification are hereby incorporated herein by reference.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Figure 1 shows the expression of plexinB1 in LNCaP, PC3 and DU145 cells using quantitative RT-PCR. Real time RT-PCR was performed on a Taqman 7700 using  $\beta$  actin template as control, average of 3 RNA extractions.
- 10 Figure 2 shows the number of colonies of  $\geq 200 \mu\text{M}$  produced after 3 weeks in 0.3% agarose by cells transfected with plexinB1 (A5359G), plexinB1 (A5653G), plexinB1 (T5714C), wild type plexinB1, empty vector or parental NIH3T3 cells. The bars indicate the mean number of colonies produced by 2
- 15 independent clones of each construct.

Figure 3 shows sequencing data for plexin B1 prostate cancer mutations.

- Figure 3a: C5060T (pro1597Leu): i) normal sequence (SEQ ID NO:1), ii) SSCP band (SEQ ID NO:2) and iii) direct sequence (SEQ ID NO:3) of tumour sample.
- 20

Figure3b: C5059 (Pro1597Ser) SSCP band (SEQ ID NO:4).

- Figure 3c: G5074A (Gly1602Thr) complementary sequence: i) normal sequence (SEQ ID NO:5), ii) SSCP band (SEQ ID NO:6) and
- 25 iii) direct sequence (SEQ ID NO:5) of tumour sample.

Figure 3d: A5359G (Thr1697Ala): i) normal sequence (SEQ ID NO:7) and ii) direct sequence (SEQ ID NO:8) of tumour sample.

- Figure 3e: T5401A (Phe1711Ile) complementary sequence: i) normal sequence (SEQ ID NO:9) and ii) SSCP band (SEQ ID
- 30 NO:10).

Figure 3f: C5468T (Thr1733Ile): i) normal sequence (SEQ ID NO:11), ii) SSCP band (SEQ ID NO:12) and iii) direct sequence (SEQ ID NO:12) of tumour sample.

5 Figure 3g: A5474G (Asn1735Ser): i) normal sequence (SEQ ID NO:13) and ii) direct sequence (SEQ ID NO:14) of tumour sample.

Figure 3h: A5596G (Thr1774Ala): i) normal sequence (SEQ ID NO:15) and ii) SSCP band (SEQ ID NO:15)

10 Figure 3i: A5653G (Thr1795Ala): i) normal sequence (SEQ ID NO:16) , ii) SSCP band (SEQ ID NO:17) and iii) direct sequence (SEQ ID NO:16) of tumour sample.

Figure 3j: C5662T (Pro1798Ser): i) normal sequence (SEQ ID NO:18) , ii) SSCP band (SEQ ID NO:19) and iii) direct sequence (SEQ ID NO:20) of tumour sample.

15 Figure 3k: A5674G (Thr1802Ala): i) normal sequence (SEQ ID NO:21) and ii) SSCP band (SEQ ID NO:22) .

Figure 3l: T5714C (Leu1815Pro) complementary sequences: i) normal sequence (SEQ ID NO:23) , ii) SSCP band (SEQ ID NO:24) and iii) direct sequence (SEQ ID NO:25) of tumour sample.

20 Figure 3m: C5980T (Arg1904Trp) complementary sequences: i) normal sequence (SEQ ID NO:26) , ii) SSCP band (SEQ ID NO:27) and iii) direct sequence (SEQ ID NO:27) of tumour sample.

Figure 4 shows the sequencing of PCR products from 7 primary  
25 prostate cancers. The arrow marks the position of the A5653G (Thr1795Ala) mutation (SEQ ID NO:16).

Figure 5 shows an alignment of plexinB1 sequences with positions of the mutations shown.

SEQ ID NOs:

	1593- 1605	1693- 1701	1707- 1715	1724- 1738	1772- 1779	1791- 1805	1811- 1819	1899- 1908
HsPLXNB1	28	37	44	50	60	69	80	89
Mm plxnb1	32	37	44	50	62	73	80	89
Dm plexB	33	39	46	55	63	74	83	91
Ce plx-2	34	40	47	56	64	75	84	
Hs PLXNB3	35	41	48	57	65	76	85	92
Hs PLXB2		42	49	58	66	77	86	93
Hs PLXNA2	36	43		59	67	78	87	110
Hs PLXNA1				59	68	79	88	110
1579 L	29							
1579 S	30							
1602 T	31							
1697 A		38						
1711 I			45					
1728 S				51				
1728 T				52				
1728 I				53				
1728 S				54				
1776 A					61			
1795 A						70		
1795 S						71		
1795 A						72		
1815 P							81	
1815 F							82	
1904 W								90

- 5 Figure 6 shows the sequencing data for the breast cancer mutations.
- Figure 6a. T5059C (pro1597Ser) complementary sequences: i) normal sequence (SEQ ID NO:95) and ii) SSCP band (SEQ ID NO:96).
- 10 Figure 6b. C5060T (Pro1597Leu) complementary sequence: i) SSCP band (SEQ ID NO:97) .
- Figure 6c. G5424A (Gly1728Ser) complementary sequences: i) normal sequence (SEQ ID NO:98) and ii)\_SSCP band (SEQ ID NO:99).
- 15 Figure 6d. G5458A (Ala1730Thr) complementary sequences: i) normal sequence (SEQ ID NO:100) and ii) SSCP band (SEQ ID NO:101) .
- Figure 6e. C5713T (Leu1815Phe) complementary sequences: i) normal sequence (SEQ ID NO:102) and ii)\_SSCP band (SEQ ID
- 20 NO:103) .

Table 1 shows plexinB1 mutations in prostate cancer.

Table 2 shows plexinB1 mutations in breast cancer.

## Experimental

### *Materials and Methods*

#### Sample Preparation

Paraffin sections were cut from 95 cases of primary prostate  
5 cancer treated by radical prostatectomy and 11 metastatic  
cancers from patients who had relapsed following hormone  
treatment. Areas of cancer were identified and microdissected  
from the sections or obtained by laser microdissection,  
depending on the size of the cancer foci. PCR products were  
10 sequenced directly by cycle sequencing (4pmole primer,  
Amplitaq, ABI)

#### In vitro mutagenesis

The A5359G, A5653G, and T5714C sequence changes  
were introduced using Quickchange kit (Stratagene) with the  
15 following primers:

GTCCATCTGTCTGTATGCCTTCGTGAGGGTGAG (SEQ ID NO:104) and  
CTCACCCCTCACGAAGGCATACAGACAGATGGAC (SEQ ID NO:105) ,  
GGAGTGCCTCTCGCCCAGCGGCCAGACCCTCG (SEQ ID NO:106) and  
CGAGGGTCTGGCCGCTGGGCGAGAGGCACTCC (SEQ ID NO:107) ,  
20 GGTGGCCGGGCACCCATTCTTTCTGACGAGG (SEQ ID NO:108) and  
CCTCGTCAGAAAGAATGGGGTGCCCGGCCACC (SEQ ID NO:109).

The sequence change was confirmed by sequencing. No other  
sequence change was seen in the cytoplasmic domain.

#### Transfection

25 NIH3T3 cells were transfected with pcDNA3-PlexB1(A5359G),  
pcDNA3-PlexB1(A5653G), pcDNA3-PlexB1(T5714C), pcDNA-PlexB1  
and pcDNA3 (vector only) by calcium phosphate transfection  
and selected with G418.

### Cell culture

LNCaP, PC3 and DU145 were grown in RPMI-1640 with 10%FCS, NIH3T3 was grown in DMEM with 10%NCS.

### Anchorage independent assays

- 5 1000 cells were grown in 12 well plates in 0.35% agarose over a layer of 0.7% agarose in medium.

### In vivo tumourigenesis assays

- NIH3T3 transfected with mutant, wild type or empty vector or parental NIH3T3 cells were injected subcutaneously into  
10 athymic nude mice.

### Screening for Polymorphisms

Control DNA from unrelated individuals of Caucasian origin was screened for the presence of identified mutations by

- 15 PCR and restriction enzyme digestion (NEB) using HaeIII (P1597L, P1597S, A1730T), AvaII (P1597S), StyI (G1602T), BstZ171 (T1697A), BclI (F1711I), BstNI (G1728S), StyI (T1733I), BsrBI (N1735S), HgaI (T1776A), HphI (T1795A), HaeIII (P1798S), HhaI (T1802A), MnlI (L1815P, L1815F) and  
20 TaqI (R1904W). Primers with a single base pair mismatch were used for StyI, BsrBI, MnlI and TaqI. The T1795A mutation destroys a HphI site. Amplified products were digested with HphI, HphI-resistant DNA bands excised from the gel, sequenced and the sequence change identified.

25

SSCP analysis was performed as previously described (Williamson, M. P. et al Genes Chromosomes Cancer 92 (1994): 108-18).

## Results

### Initial RT-PCR Screen

RT-PCR of RNA from prostate cancer cell lines showed that plexins A1, A3 and B1 were expressed in prostate cells. The cDNA of the cytoplasmic domains of plexin A1, A3 and B1 was sequenced in the prostate cancer cell lines PC3, DU145, LNCaP and benign prostate cell line Pre2.8.

A single nucleotide change (A to G) was observed in plexin B1 in LNCaP cDNA at nucleotide 5359, which potentially changes threonine 1697 to an alanine. The sequence change is heterozygous in LNCaP cells. Karyotyping of LNCaP has revealed that this cell line has three copies of chromosome 3p. Estimation of the relative intensities of the mutant and wild type bands indicated that the mutation was present in two of the three copies of chromosome 3p. PlexinB1 is overexpressed in LNCaP as shown by quantitative RT-PCR (Figure 1).

The sequence change was absent from 120 control chromosomes of similar genetic background to LNCaP.

DNA from 25 prostate tumours was extracted from paraffin sections and sequenced directly for the mutation found in LNCaP. The Thr1697Ala mutation was found in three of the cancers, but was absent from seminal vesicle or lymph node tissue extracted from the same patient in the two cases where such tissue was available. This sequence change therefore represents a somatic change associated with prostate cancer.



### Additional Mutations

80 primary prostate tumours (including 10 of the 25 providing DNA for sequencing the mutation seen in LNCaP) and 11 prostate cancer metastases were screened for mutations in the cytoplasmic domain of the plexinB1 gene by SSCP analysis. Aberrant bands were excised from the gel and sequenced directly. 12 additional mis-sense mutations were identified (Table 1, Figure 3).

One of these mutations, A5653G, which potentially changes the threonine at position 1795 to an alanine, was found in 7/11 (64%) of metastases and 33/80 (41%) of primary cancers. The presence of the T1795A mutation was confirmed by direct sequencing of DNA from the metastases (Figure 4) and by restriction enzyme digestion in the 33 primary tumours.

Position 1795 is part of a potential serine/threonine kinase phosphorylation site which is highly conserved in evolution. A threonine or serine is present at this position in the protein in mouse, *Drosophila* and *C.elegans* plexinB1 homologues as well as members of the other classes of the plexin family of proteins (plexins A1, A2, A3, B2, B3 and D1) (Figure 5).

A T5714C sequence change, predicted to result in a change of amino acid from leucine 1815 to proline was found in 3 metastases. A leucine or valine in this position is conserved in plexinB1 homologues in other species and in other plexin family members (Figure 5).

A C5060T mutation, which potentially changes proline 1597 to leucine, was also found in 3 metastases and a second mutation in a primary tumour (C5059T) changes the same amino acid to serine. A proline at this position in the protein is not conserved during evolution (Figure 5)).

Six further mutations were identified in single metastases: G5074A (Gly1602T), T5468C (Thr1733Ile), A5474G (Asn1735Ser), C5662T (Pro1798Ser), A5674G (Thr1802Ala) and C5980T (Arg1904Trp). Thr1733 and Pro1798 are conserved in both A and B class plexins, while Gly1602, Asn1735, Arg1904 are conserved in class B plexins. Thr1802 is replaced with acidic amino acids in A class plexins which may mimic a phosphorylation site. Finally two mutations (F1711I and T1776A) were found in single primary tumours. Phe1711 is conserved in class B plexins and Thr1776 is conserved in class A and B plexins.

All 12 additional mutations were found to be absent from normal tissue of the same patient where available and are therefore somatic. The sequence changes were absent from at least 50 control individuals in every case.

The present findings show that mutation of the plexinB1 gene is associated with prostate cancer progression. The incidence of mutations was higher in prostate cancer metastases than primary tumours, with mutations in 91% of metastases (10/11) and 45% (36/80) of primary tumours. The metastases frequently contained multiple mutations. Three of the metastases each contained 3 mutations within this short stretch of the

plexinB1 gene and 3 others contained two mutations. 8 of the mutations (L1815P in 3 tumours and P1798L in 3 tumours, T1802A and Pro1798Ser in one tumour each) occurred in addition to the common Thr1795Ala change. T1802A was found in the same SSCP band as the common (T1795A) mutation indicating that the two mutations are on the same allele. In contrast, only single mutations were found in primary tumours. In addition, the proportion of cells containing mutant plexinB1 was higher in metastases than primary tumour tissue. In one case where both primary and metastatic tissues were available from the same patient, the mutant allele was more highly represented in the metastases than the primary tumour. Together these results provide indication that mutations in the plexinB1 gene are selected for during cancer metastasis.

#### PlexinB1 Mutations in Breast Cancer

30 breast cancer lymph node metastases were screened for mutations in exons 22-29 of the plexin B1 gene by SSCP and 7 mutations were found in 6 cases (Table 2, Figure 6). Three metastases had a T5059C sequence change, which is predicted to alter Pro1597 to Ser, and one metastasis had a mutation in the adjacent nucleotide (C5060T), which alters the same amino acid to Leu. Both these mutations were also observed in prostate cancer metastases. One breast cancer metastasis had the sequence change C1713T, which changes Leu1815 to Phe. Leu1815 was altered to Pro in 3 cases of prostate cancer metastasis. Two of the mutations were only found in breast cancer metastasis: G5452A (Gly1728Ser) and G5458A (Ala1730Thr). G5452A and C1713T were found in the same

metastasis. These mutations were not present in 100 control chromosomes. These mutations were present at a low copy number in the lymph node metastasis.

Nucleotide	Amino acid	somatic +'	primary(95)	metastases(11)										Total
				1	2	3	4	5	6	7	8	9	10	
C5060T	P1597L	X			X	X		X	X					4
C5059T	P1597S	ND	1											1
G5074A	G1602T	X							X					1
A5359G*	T1697A*	X	3											3
T5401A	F1711I	ND	1											1
C5468T	T1733I	X								X				1
A5474G	N1735S	X								X				1
A5596G	T1776A	ND	1											1
A5653G	T1795A	X	33	X	X	X	X	X			X	X		40
C5662T	P1798S	ND										X		1
A5674G	T1802A	X				X								1
T5714C	L1815P	X			X			X			X			3
C5980T	R1904W	ND											X	1
			(39/95=41%)	(10/11=91%)										59

\* T1697A mutation was found in LNCaP and 3 primary tumours by direct sequencing  
 +' mutation absent from non cancer tissue from the same patient

Table 1

		metastases(30)						Total
		1	2	3	4	5	6	
T5059C	P1597S	x	x	x				3
C5060T	P1597L				x			1
C5713T	L1815F					x		1
G5452A	G1728S					x		1
G5458A	A1730T						x	1
								7

5

Table 2

		metastases(30)						Total
		1	2	3	4	5	6	
T5059C	P1597S	x	x	x				3
C5060T	P1597L				x			1
C5713T	L1815F					x		1
G5452A	G1728S					x		1
G5458A	A1730T						x	1
								7